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Characterization of Attenuated Strains of Rift Valley Fever Virus

By CYNTHIA A. ROSSI* AND MICHAEL J. TURELL

Department of Arboviral Entomology, Disease Assessment Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701-5011, U.S.A.

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SUMMARY

The wild-type ZH501 strain of Rift Valley fever (RVF) virus and two small-plaque strains (T1 and T46) derived from it were characterized by plaque size, pathogenicity for hamsters and ability to replicate in Vero cells. Additionally, a mutagenized, attenuated, large-plaque, vaccine-candidate strain of RVF virus (ZH548-M12) was also studied. Infections with either the ZH501 or T46 strain were uniformly fatal to hamsters. In contrast, nearly all hamsters infected with either the T1 or ZH548-M12 strains survived and were immune to challenge with 10^5 LD_{50} of the ZH501 strain. Both of these attenuated strains failed to replicate in Vero cells maintained at 41 °C, whereas the more virulent strains (ZH501 and T46) replicated at this temperature. The low virulence and ability to induce protection against lethal RVF virus challenge that is associated with the T1 and ZH548-M12 strains make them potential vaccine candidates.

INTRODUCTION

All strains of Rift Valley fever (RVF) virus tested have produced plaques in a number of cell culture systems. Many of these strains produce plaques of non-uniform size in various cell systems. This variability in plaque size suggests that other properties, such as virulence, might also be heterogeneous. By repeated plaque isolation, many stocks of small- and large-plaque variants have been produced (Boyle, 1967; Moussa *et al.*, 1982; Peters & Anderson, 1981; Caplen *et al.*, 1985). To date, no consistent relationship between virulence and plaque size has been found for these strains. The present study characterizes two small-plaque mutants which were selected because of the large percentage of uniformly small plaques formed when initially assayed. These were compared with wild-type ZH501 and a large-plaque, attenuated, vaccine-candidate strain, ZH548-M12. We conducted tests to determine the infectivity and virulence of these strains for hamsters and studied their growth characteristics in Vero cells at various incubation temperatures.

METHODS

Virus. The ZH501 strain was isolated from the serum of a fatal human haemorrhagic fever victim in Egypt, during an epidemic in 1977 (Meegan, 1979). This strain was passaged twice in foetal rhesus monkey lung (FRhL) cells. The titre of this stock virus was $10^{7.7}$ p.f.u./ml as measured by plaque assay on Vero cell monolayers (Vero 76 cells were received from the Centers for Disease Control at passage level 135 and used at passage levels 143 to 152).

During the course of vector competence studies, numerous individual mosquito specimens were assayed for RVF virus. Nearly all of these isolates produced primarily large plaques. Several specimens were unusual in that they produced small plaques. Two of these, T1 and T46, were selected for additional study.

The T1 strain was recovered from a female *Culex pipiens* mosquito and produced uniformly small plaques with a titre of $10^{5.5}$ p.f.u./ml. This mosquito had been inoculated 7 days previously with approximately 0.9 p.f.u. of virus recovered from another *C. pipiens* that had fed on a viraemic hamster. The hamster had been inoculated intraperitoneally (i.p.) with the ZH501 strain of RVF virus. Thus, the passage history of the T1 strain was ZH501, $FRhL_2$, $hamster_1$, $mosquito_2$.

The T46 strain was isolated from an *Aedes taeniorhynchus* mosquito that had fed on an RVF (ZH501) virus-infected gerbil. T46 produced predominantly small plaques with a titre of $10^{6.6}$ p.f.u./ml. Thus, the passage history of T46 was ZH501, $FRhL_2$, $gerbil_1$, $mosquito_1$.

The ZH548-M12 strain, a potential RVF virus vaccine strain, was derived from the ZH548 strain by 12 successive cycles of replication in MRC5 cells and mutagenization with 5-fluorouracil (5-FU) (Caplen *et al.*, 1985). This strain is attenuated for adult mice (Caplen *et al.*, 1985) and pregnant ewes (Morrill *et al.*, 1987). The ZH548 strain was originally isolated from the serum of an uncomplicated human febrile case of RVF that occurred in the same area of Egypt as the fatal case that produced the ZH501 strain. ZH548-M12 was passaged twice in suckling mice (SM) and once in FRhL cells prior to mutagenesis and once in Vero cells after mutagenesis. Thus, its passage history was ZH548, SM_2, $FRhL_1$, $(MRC5\text{-}5FU_1\ MRC5_2)_{12}$, $Vero_1$.

Production of stock viruses. Strains T1 and T46 were plaque-purified by inoculating serial 10-fold dilutions onto Vero cell monolayers. A single small plaque was picked with a sterile Pasteur pipette, placed in a test tube containing 1 ml of diluent (10% calf serum in Medium 199 with Hanks' salts and 0·075% $NaHCO_3$ plus 100 units penicillin, 100 μg streptomycin, 50 μg gentamicin and 5 μg fungizone/ml) and stored at −70 °C. After two repetitions of this procedure only small plaques were detected in each of the strains. The parental strain, ZH501, and the ZH548-M12 strain also were passaged twice in Vero cells to provide a comparable passage history. After passage in Vero cells, the ZH501 strain continued to produce variable-sized plaques with approximately 70% having diameters of 0·2 cm or above; in contrast the ZH548-M12 strain produced consistently large plaques.

The titre of each of the viral strains was then amplified by an additional passage in Vero cells (m.o.i. 0·01). Instead of the solid overlay normally used for plaque assays, 40 ml of maintenance medium (5% foetal bovine serum in Earle's minimum essential medium containing non-essential amino acids and L-glutamine plus 100 units penicillin, 100 μg streptomycin, and 50 μg gentamicin/ml) was added to each 75 cm^2 flask after 1 h adsorption. After 72 h, the contents of the flask were frozen at −20 °C for 10 min and then thawed. An additional 10% foetal bovine serum was added to the medium which was then divided into 1 ml samples and stored at −70 °C until use. The identity of each of the strains used in this study was confirmed as RVF virus by a plaque-reduction neutralization test (PRNT) (Earley *et al.*, 1967) using antibody produced against ZH501.

Viral replication in Vero cells. Vero cells which had been grown to confluence in 75 cm^2 flasks were drained of maintenance medium and inoculated with 1 ml containing approximately $10^{3.9}$ p.f.u. (m.o.i. 0·01) of each of the viral suspensions. After 1 h adsorption at 35 °C, the monolayers were washed, maintenance medium (40 ml) was added to each flask and the flasks were incubated at 35(±1) °C or 41 (±1) °C. Samples (1 ml) of supernatant were frozen at −70 °C at various time intervals to monitor extracellular viral titres.

Infection of hamsters. Susceptible female golden Syrian hamsters (*Mesocricetus auratus*) were inoculated i.p. (0·2 ml/hamster) with serial 10-fold dilutions of the various strains. Hamsters were caged individually and observed twice daily for signs of illness and the time before death was recorded. Livers and brains were removed aseptically from dead hamsters to confirm the cause of death. A portion of the liver or brain was triturated in diluent to prepare a 10% (w/v) suspension and frozen at −70 °C until assayed on Vero cells. All surviving hamsters were challenged i.p. with $10^{4.0}$ p.f.u. ($10^{5.0}$ LD_{50}) of the ZH501 viral strain. Infection with this strain of RVF virus is nearly always fatal to hamsters (Gargan *et al.*, 1983). Therefore, hamsters surviving challenge with ZH501 virus were considered to have been infected by the original exposure. Sera were obtained from surviving hamsters and assayed for RVF virus antibody. The Reed-Muench formula (Reed & Muench, 1938) was used to determine ID_{50} and LD_{50}.

Viraemia profiles and antibody production. Viraemia profiles of the various strains were determined in hamsters after i.p. inoculation of approx. $10^{2.6}$ p.f.u. in 0·2 ml of diluent. Liver tissues were removed from all dead hamsters to confirm the cause of death. To monitor antibody production, we obtained sera from surviving hamsters that had been inoculated with the various RVF strains.

Virus assays. We evaluated samples for infectious virus by a standard plaque assay in Vero cells (Gargan *et al.*, 1983). Cells were incubated at 35 °C and stained with a 0·015% neutral red solution 4 days after inoculation. To monitor plaque size, a minimum of 300 plaques of each viral strain were measured with a micrometer. Viral identity was confirmed by polyclonal, sheep anti-RVF virus antibodies in a PRNT. The PRNT was also used to quantify the antibody response in hamsters infected with any of the strains tested.

Reversion studies. During the above studies, the various strains of RVF virus were monitored for signs of reversion as evidenced by a change in plaque size or a change in virulence for hamsters. Representative samples of hamster- and tissue culture-passaged viral strains were inoculated i.p. into hamsters to monitor virulence. In addition, the T1 and ZH548-M12 strains were inoculated i.p. into two hamsters. These hamsters were killed and their livers removed 2 and 3 days after virus inoculation. The liver suspension containing the higher virus titre was then inoculated into a second pair of hamsters; this procedure was continued until three liver passages had been completed. Virus recovered from the third liver passage was inoculated i.p. into 25 susceptible hamsters (0·1 ml containing 10^4 p.f.u.) to evaluate possible reversion to a move virulent form. Hamsters were monitored as previously described for 30 days. All surviving hamsters were challenged i.p. with $10^{5.0}$ LD_{50} of the ZH501 strain of RVF virus.

RESULTS

Plaque characteristics and virus identity

The range in plaque sizes for the virus strains when assayed on Vero cells at 35 °C and stained with neutral red 4 days after inoculation are shown in Fig. 1. The ZH501 strain of RVF virus produced plaques of various sizes (mean ± standard deviation 0·21 ± 0·07 cm). Approximately 70% of the plaques were ⩾ 0·2 cm in diameter, while the remaining plaques were smaller. The T46 strain originally produced 90 to 95% small plaques with an occasional large plaque. However, after plaque purification, only small plaques were detected (0·13 ± 0·01 cm). The T1 strain produced exclusively small plaques upon initial isolation. The mean plaque size for this strain was 0·14 (± 0·02) cm. The ZH548-M12 strain produced large plaques (0·38 ± 0·06 cm). All strains were neutralized by 80% or more by polyclonal, sheep anti-RVF (ZH501) virus antibodies.

Virus replication in Vero cells

After inoculation with approximately $10^{3.9}$ p.f.u. (m.o.i. 0·01) and incubation at 35 °C, complete cell destruction was noticeable (4+ c.p.e.) by day 4 for both the ZH501 and T46 viral strains. In contrast, T1 and ZH548-M12 replication caused less cell damage (2+ and 3+ c.p.e., respectively) by day 4 and there were viable cells for as long as 10 days after inoculation. The patterns of viral replication were similar for all four viral strains, with titres of approximately 10^7 p.f.u./ml reached at 4 to 5 days post-infection (Table 1).

At elevated temperature (41 °C), the ZH501 and T46 strains continued to replicate well, with complete cell destruction occurring by 3 to 4 days after infection. In contrast, the T1 and ZH548-M12 strains failed to replicate in Vero cells incubated at 41 °C and were undetectable after 3 days (Table 1).

ID_{50} and LD_{50} in hamsters

The ZH501, T1 and T46 strains were highly infectious for adult hamsters (ID_{50} 0·1 to 0·3 p.f.u.) (Table 2), but the ZH548-M12 strain was approximately 200-fold less infectious (ID_{50} 40 p.f.u.). Both the ZH501 and T46 strains were highly virulent, with no hamsters surviving infection with either strain Virus recovered from livers of hamsters dying after inoculation with the T46 strain produced uniformly small plaques. In contrast, both the T1 and ZH548-M12 strains were highly attenuated, and we could not determine an LD_{50} for either of these two strains (Table 2).

Viraemia profile and mean time between infection and death

After inoculation of hamsters ($10^{2.6}$ p.f.u.) with either the T46 or ZH501 strains, there was a rapid rise in viraemia that peaked at $10^{7.6}$ p.f.u./ml and all hamsters infected with either of these strains died in ⩽ 4 days (Fig. 2, Table 2). The T1 strain produced a much lower viraemia in hamsters. Viraemia titres peaked at 48 h ($10^{3.6}$ p.f.u./ml) and were undetectable by 64 h. None of the T1-infected hamsters used for the comparison of viraemias died of an RVF viral infection but one (1%) of the 77 hamsters infected with the T1 strain during our study died. All surviving hamsters were immune to challenge with ZH501. After inoculation with the ZH548-M12 strain, only one of five hamsters developed a detectable viraemia ($10^{2.2}$ p.f.u./ml). This hamster died 2 days later (day 5). In contrast, the four hamsters that failed to produce a detectable viraemia survived and were immune to challenge with ZH501. It is possible that this hamster died due to the stress of repeated bleedings and handling, rather than from its RVF virus infection. However, in addition to this hamster, six other hamsters died between 11 and 17 days after i.p. inoculation with $10^{2.2}$ or more p.f.u. of the ZH548-M12 strain. As four of these hamsters were not bled or handled, these deaths should be attributed to viral infection. Although virus was not recovered from blood specimens taken on day 7 from two of these hamsters, or from liver suspensions from any of these hamsters on autopsy, it was recovered from brain specimens (mean titre $10^{8.1}$ p.f.u./g of brain tissue). Thus, a total of seven (14%) of the 49 hamsters inoculated with the ZH548-M12 strain in these studies died.

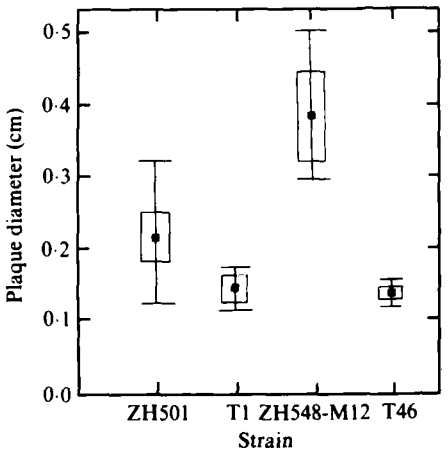

Fig. 1

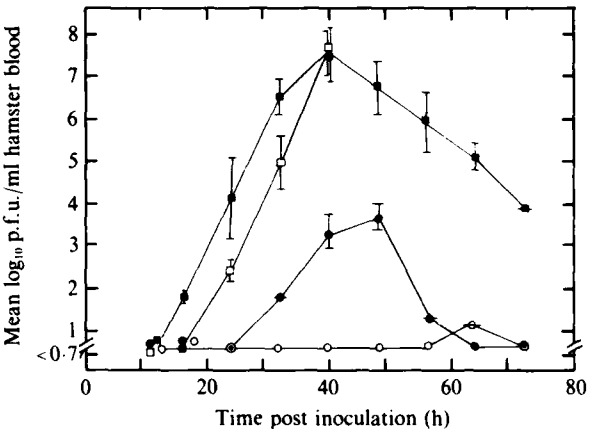

Fig. 2

Fig. 1. Plaque size of RVF virus strains assayed on Vero cells. Cells were incubated at 35 °C for 4 days after inoculation before being stained. □ indicates the standard deviation about the mean (■). The vertical bar represents the range of values obtained for plaque diameter.

Fig. 2. Replication of strains ZH548-M12 (○), ZH501 (■), T1 (●) and T46 (□) in hamsters after i.p. inoculation of $10^{2.6}$ p.f.u. Titres represent p.f.u./ml of hamster blood. The vertical bar indicates the standard deviation.

Table 1. *Replication of selected strains of Rift Valley fever virus in Vero cells*

	Incubated at 35 (±1) °C			Incubated at 41 (±1) °C		
Strain	C.p.e	Time to peak titre (days)	Peak titre ($\log_{10}$ p.f.u./ml)	C.p.e.	Time to peak titre (days)	Peak titre ($\log_{10}$ p.f.u./ml)
ZH501	4+	5	7·1	4+	4	6·8
T46	4+	5	7·0	4+	4	6·8
T1	2+	5	7·1	0	NA*	<0·7
ZH548-M12	3+	5	6·8	0	NA*	<0·7

*NA, Not applicable.

Table 2. *Infectivity for hamsters of selected strains of Rift Valley fever virus*

Strain	ID_{50} (p.f.u.)	LD_{50} (p.f.u.)	ID_{50}/LD_{50}	Mortality in infected hamsters (%)*	Mean time before death (h) and range
ZH501	0·1	0·1	1	100 (38/38)	57 (48–84)
T46	0·3	0·3	1	100 (40/40)	44 (40–64)
T1	0·1	>630000	$\geqslant 10^{6.8}$	1 (1/77)	72 (NA)†
ZH548-M12	40·0	>1600000	$\geqslant 10^{4.6}$	14 (7/49)	288 (120–408)

* Includes five or ten hamsters bled by cardiac puncture. Mortality rates were not significantly different for bled or non-bled hamsters. Hamsters were inoculated with $\geqslant 10^{2.6}$ p.f.u. of virus.

† NA; not applicable; titres could not be detected 3 days post-inoculation.

Antibody production

Serum neutralizing antibodies were detectable at a 1 : 10 dilution 8 days after inoculation with the T1 strain but not until day 19 for hamsters inoculated with the ZH548-M12 strain (Table 3). The T1 strain induced the highest antibody titres at each time interval tested. Antibody titres for the T1 and ZH548-M12 strains continued to rise with increasing time and reached median titres of 1 : 2560 and 1 : 160, respectively, 24 days after inoculation.

Table 3. *Neutralizing antibody in Rift Valley fever virus-infected hamsters*

Strain		Time post-inoculation (days) 8	12	19	24
T1	No. tested	5	5	7	7
	GMT*	7	17	580	1902
	Range	<10–10	10–40	320–1280	1280–2560
	% ⩾ 10	40	100	100	100
ZH548-M12	No. tested	4	4	3	3
	GMT*	<10	<10	50	202
	Range	<10	<10	40–80	160–320
	% ⩾ 10	0	0	100	100

* Geometric means of the reciprocal of the lowest dilution giving at least a 50% reduction in the number of plaques on Vero cells.

Reversion studies

No significant change in the plaque size of any of the strains of virus was observed on Vero cells after either a single passage in Vero cells or hamsters. The T1 and ZH548-M12 strains were further tested for reversion by inoculating hamsters with the virus and noting any changes in its virulence. All hamsters (19/19) survived exposure to samples that had been passaged once either in Vero cells incubated at various temperatures or in hamsters. The T1 strain remained avirulent (0/25 dead) after three consecutive liver passages in hamsters. All of these hamsters survived challenge with ZH501. Similarly, there was no significant difference in mortality (chi-square test, $P > 0{\cdot}3$) in hamsters inoculated with either the stock ZH548-M12 virus (7/49, 14%) or the liver-passaged virus (5/17, 29%). Again, deaths associated with the ZH548-M12 strain appeared to be due to encephalitis.

DISCUSSION

The strains of RVF virus used in this study differed significantly in their infectivity and virulence for hamsters as well as in their ability to replicate at 41 °C in Vero cells. However, these differences did not correlate with plaque size. Other studies attempting to correlate virulence of RVF virus with plaque size have also had inconsistent results. For example, Moussa *et al.* (1982) isolated a small-plaque variant of RVF virus from the virulent ZH501 strain originally producing both large and small plaques. This small-plaque variant demonstrated reduced virulence for adult mice and rats. However, Caplen *et al.* (1985) described a large-plaque variant of another Egyptian RVF virus strain that was attenuated for adult mice, hamsters and pregnant ewes. Boyle (1967) reported a small-plaque variant that was more virulent for adult mice than a large-plaque variant. Our study also found no direct relationship between pathogenicity and plaque size for RVF virus. The two small-plaque variants of RVF virus (T1 and T46) exhibited a wide range of virulence for hamsters. The T46 strain was at least as virulent as its parent strain, ZH501. All infections with either of these strains were lethal to hamsters. However, the T1 strain was highly attenuated for hamsters. Even with the highest dose of T1 administered ($10^{5.8}$ p.f.u.), nine out of ten hamsters survived. Thus, because the ID_{50} for this strain was 10^{-1} p.f.u., the LD_{50} contained at least $10^{6.8}$ ID_{50} units. The large-plaque ZH548-M12 strain was also greatly attenuated for hamsters. At the highest dose administered, $10^{6.2}$ p.f.u., all six hamsters survived. Because the ID_{50} for this strain in hamsters was $10^{1.6}$ p.f.u., the LD_{50} contained at least $10^{4.6}$ ID_{50} units. The large safety margins indicate that these two strains may have promise as live, attenuated vaccines. However, sporadic hamster deaths (7/49, 14%) were associated with infection with the ZH548-M12 strain.

The reduced virulence of these strains (T1 and ZH548-M12) in hamsters may have been due to their decreased ability to replicate efficiently in hamsters (i.e., low viraemia titres were produced). This may have been due to any of several defects in the viruses or might be specifically related to their temperature sensitivity. Both of these strains replicated well and

produced detectable c.p.e. in Vero cells when grown at 35 °C, but, when incubated at 41 °C, they did not replicate or produce any detectable c.p.e. Normal body temperature in a hamster is between 36·2 and 37·5 °C (van Hoosier & Ladiges, 1984), and the T1 strain replicates less efficiently at 37 °C than at 35 °C (J. Smith, personal communication). Thus, viral replication in a hamster may be sufficiently reduced to allow the hamster's immune system to clear the infection prior to irreversible pathological changes. In contrast, the two highly virulent strains of RVF virus (ZH501 and T46) did not show any signs of temperature sensitivity and replicated to high titres in hamsters.

The mean time to death in hamsters inoculated with either of the two highly virulent strains was inversely related to the dose inoculated (data not shown). Most hamsters infected with approximately $10^{2.6}$ p.f.u. of virus died 2 to 3 days after inoculation. One of the hamsters inoculated with the ZH548-M12 strain died 5 days after infection and six others died between 11 and 17 days post-infection. These latter deaths were typical of RVF encephalitis in that they occurred after 9 days; virus was recovered from brain, but not liver, specimens and hamsters had hind leg paralysis on the day before death.

Due to the expectation of continued epizootics of RVF throughout Africa, and the possibility of dissemination of RVF virus outside Africa, a safe, effective vaccine suitable for immunizing both man and animals needs to be developed. The time required to produced immunity suggests that inactivated vaccines might not prove effective. Two matters of concern when working with live, attenuated vaccines are whether or not the vaccine strain will revert to the characteristics of the parent strain and whether or not the vaccine is abortifacient or teratogenic. Both abortions and lambs dying shortly after birth have been observed when pregnant ewes were immunized with the live, attenuated Smithburn neurotropic strain (Smithburn, 1949; Coetzer & Barnard, 1977; Weiss, 1962). This has major implications for an immunization programme. We monitored reversion of the T1 strain of RVF virus by examining plaque size in Vero cells and virulence for hamsters. Neither parameter changed during the course of these experiments. Thus, preliminary evidence indicates that the T1 strain should be considered as a possible candidate for a live, attenuated veterinary RVF vaccine.

These investigations were performed in partial fulfilment of the MS degree program at Hood College. In conducting the research described in this report, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals*, as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The views of the authors do not purport to reflect the positions of the Department of the Army, the Department of Defense, or Hood College.

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